

Improvement of detection sensitivity of T-2 and HT-2 toxins using different fluorescent labeling reagents by high-performance liquid chromatography[☆]

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Received 17 May 2007; received in revised form 27 September 2007; accepted 28 September 2007

Available online 2 October 2007

Abstract

T-2 and HT-2 toxins are *Fusarium* mycotoxins that can occur in cereals and cereal-based products. Three fluorescent labeling reagents, i.e. 1-naphthoyl chloride (1-NC), 2-naphthoyl chloride (2-NC) and pyrene-1-carbonyl cyanide (PCC), were used for the determination of T-2 and HT-2 toxins by high-performance liquid chromatography (HPLC) with fluorescence detection (FD). Pre-column derivatization of T-2 and HT-2 toxins was carried out under mild conditions (50 °C, 10 min) in toluene with 4-dimethylaminopyridine (DMAP) as catalyst. All fluorescent derivatives were identified and characterized by HPLC-tandem mass spectrometry (HPLC-MS/MS). Optimal stoichiometric ratios (toxin:derivatizing reagent:catalyst), linear range and repeatability of the reaction, stability and sensitivity of the derivatives were determined. A wide linear range (10–1000 ng of either derivatized T-2 or HT-2 toxin), good stability (up to 2 weeks at –20 °C or 5 days at room temperature) of the fluorescent derivatives and good repeatability of the reaction (RSD ≤ 8%) were observed. Detection limits (based on a signal-to-noise ratio of 3:1) were 10.0, 6.3 and 2.0 ng for derivatized T-2 toxin and 6.3, 2.3 and 2.8 ng for derivatized HT-2 toxin with 1-NC, 2-NC and PCC, respectively. In terms of sensitivity and repeatability, PCC and 2-NC reagents showed better performance than 1-anthrolylnitrile (1-AN), a previously reported labeling reagent for T-2- and HT-2 toxins. Preliminary studies also showed the applicability of PCC and 2-NC as fluorescent labeling reagents for the simultaneous determination of T-2 and HT-2 toxins in cereal grains by HPLC/FD following immunoaffinity column clean-up.

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Keywords: 1-Naphthoyl chloride; 2-Naphthoyl chloride; Pyrene-1-carbonyl cyanide; Fluorescent labeling reagents; T-2 and HT-2 toxins

1. Introduction

T-2 and HT-2 toxins are ‘type-A’ trichothecene mycotoxins produced by several *Fusarium* species, mainly *F. sporotrichioides*, *F. poae*, *F. equiseti* and *F. acuminatum*. These mycotoxins may contaminate a variety of cereal grains, such as wheat, maize, oats, barley and rice, especially in cold climate regions or during wet storage conditions. T-2 and HT-2 toxins can also be found in cereal-based products intended for direct human consumption [1–3]. A recent data collection, aim-

ing to evaluate the risk of dietary exposure to *Fusarium* toxins by the population of EU member states, showed that T-2 and HT-2 toxins are quite common contaminants in cereals in the EU. In particular, the most frequently contaminated cereal samples were maize (28%), wheat (21%) and oats (21%) for T-2 toxin and oats (41%), maize (24%) and rye (17%) for HT-2 toxin [3].

T-2 toxin, the most toxic trichothecene, is a potent inhibitor of DNA, RNA and protein synthesis, and shows immunosuppressive and cytotoxic effects both *in vivo* and *in vitro* [1,4]. The acute toxicity of T-2 and HT-2 toxins are quite similar. *In vivo* animal experiments showed that T-2 toxin is rapidly metabolised to HT-2 toxin and, consequently, the toxicity of T-2 toxin *in vivo* might partly be attributed to HT-2 toxin [1]. Recently, the European Commission (EC) has established, with Regulations No. 856/2005 and No. 1881/2006, admissible levels of several *Fusarium* toxins in cereals and cereal-based products

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which were effective from 1 July, 2006. Maximum admissible levels for T-2 and HT-2 toxins in unprocessed cereals and cereal products are currently under discussion [5,6].

There is a need to develop sensitive and accurate analytical methods for determining these mycotoxins in cereals and cereal-based products in order to properly assess the relevant risk of human exposure. Different chromatographic methods, including gas-chromatographic (GC) and liquid-chromatographic (LC) methods coupled with mass spectrometry (MS), for the analysis of 'type-A' trichothecenes (including T-2 and HT-2) have been reported by several authors [7–12], but problems of accuracy due to matrix effects were observed [9,11]. High-performance liquid chromatography (HPLC) with UV detection is generally not applicable to 'type-A' trichothecenes for sensitive detection due to the lack of a strong chromophore group within their structures. HPLC methods for T-2 and HT-2 toxins have been developed in which derivatizing reagents are used to allow detection by fluorescence. Currently, only coumarin-3-carbonyl chloride (not commercially available) and 1-anthrolylnitrile (1-AN) have been used as fluorescent labeling reagents for some 'type-A' trichothecenes [13–17]. The latter has been applied for the simultaneous determination of T-2 and HT-2 toxins in wheat, maize and barley by HPLC with fluorescence detection (FD) following immunoaffinity column clean-up. The method allowed the determination of T-2 and HT-2 toxins at levels of 5 and 3 $\mu\text{g kg}^{-1}$ (LOD, limit of detection), respectively, with good accuracy and precision. Nevertheless, the method did not allow the determination of HT-2 toxin in oats because of interfering chromatographic peaks occurring at the retention time of the HT-2(1-AN) derivative [17].

Intake estimates indicate that the presence of T-2 and HT-2 toxins in the diet at low levels can be of some concern for human health. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the PMTDI (provisional maximum tolerable daily intake) of T-2 and HT-2 toxins, alone or in combination, at 0.06 $\mu\text{g kg}^{-1}$ body weight per day. In addition, the Committee noted that data on T-2 and HT-2 toxins in food are quite limited and of low quality and the sensitivity of the analytical methods used was poor in many cases [1]. The aim of this work was to find new commercially available fluorescent labeling reagents to improve detection sensitivity for T-2 and HT-2 toxins by HPLC/FD analysis, so that they might be used for the monitoring/surveillance of these toxins in a variety of food matrices, including oats and processed cereal products.

2. Experimental

2.1. Chemicals and materials

Acetonitrile and methanol (both HPLC grade) and toluene (for organic residue analysis) were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). T-2 toxin, HT-2 toxin, 1-naphthoyl chloride (1-NC), 2-naphthoyl chloride (2-NC), 4-dimethylaminopyridine (DMAP) were purchased from Sigma–Aldrich (Milan, Italy). Pyrene-1-carbonyl cyanide

(PCC) and 1-anthroyl cyanide (1-anthroylnitrile, 1-AN) were purchased from Wako (Neuss, Germany). T-2 immunoaffinity columns (T-2testTM HPLC) were obtained from Vicam (Watertown, MA, USA), glass microfibre filters (Whatman GF/A) and paper filters (Whatman N. 4) from Whatman (Maidstone, UK).

2.2. Preparation of standard and reagent solutions

T-2 and HT-2 toxin stock solutions were prepared by dissolving the solid commercial toxins in acetonitrile (1.00 $\mu\text{g mL}^{-1}$). T-2 and HT-2 toxin standard solutions for HPLC calibration were prepared in acetonitrile/water (70:30, v/v) by dissolving adequate amounts of the stock solution, previously evaporated to dryness under nitrogen stream. T-2 and HT-2 toxin standard solutions for all other experiments were prepared in acetonitrile by diluting aliquots of the stock solution. DMAP solutions were prepared in toluene at concentrations of 0.16, 0.33, 0.63, 0.95 and 2.00 $\mu\text{g mL}^{-1}$. Derivatizing reagent solutions were prepared in toluene at concentrations of 0.12, 0.24, 0.49, 0.73 and 1.00 $\mu\text{g mL}^{-1}$ (1-NC and 2-NC), 0.16, 0.33, 0.66 and 1.00 $\mu\text{g mL}^{-1}$ (PCC) and 0.30 $\mu\text{g mL}^{-1}$ (1-AN).

2.3. Apparatus

HPLC analyses were carried out using a ProStar system (Varian Inc., Palo Alto, CA, USA) equipped with a Rheodyne (Cotati, CA, USA) model 7125 injection valve, a fluorometric detector and the Star data system version 6.20 (Varian Inc.). The analytical column was a Phenyl-Hexyl Luna[®] (150 mm \times 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA), preceded by a SecurityGuardTM C₁₈ cartridge (4 \times 3 mm i.d., 5 μm) (Phenomenex). The flow rate of the mobile phase was 1.0 mL min⁻¹. A binary gradient was applied as follows: the initial composition of the mobile phase, 70% acetonitrile:30% water (v/v), was kept constant for 5 min, then the acetonitrile content was linearly increased to 85% in 10 min, and kept constant for 10 min. Finally, to clean the column the amount of acetonitrile was increased to 100% in 2 min and kept constant for 5 min.

2.4. Derivatization procedure

Due to its high efficiency for a variety of acylation reactions, the base DMAP was used as reaction catalyst. The derivatization procedure was performed as previously described by Visconti *et al.* [17], with minor modifications. In particular, a portion of T-2 and HT-2 toxin standard solutions (or purified cereal extract) was placed in a 4-mL screw-cap amber vial and evaporated under a stream of nitrogen gas at ca. 50 °C in a heater block. Fifty microliters of DMAP solution, followed by 50 μL of the fluorescent labeling reagent (1-AN, 1-NC, 2-NC or PCC), were added to the dried residue. The vial was closed and mixed by vortex for 1 min. After the cap was removed, the mixture was allowed to dry under a gentle stream of nitrogen for 10 min at ca. 50 °C and reconstituted with 1000 μL of a mixture acetonitrile/water (70:30, v/v). Twenty microliters of the solution were injected

into the chromatographic apparatus by a ‘full loop’ injection system.

2.5. Identification of T-2 and HT-2 derivatives

The identity of the new fluorescent derivatives [T-2(1-N); HT-2(1-N)₂; T-2(2-N); HT-2(2-N)₂; T-2(PC); HT-2(PC)₂] was confirmed by HPLC–MS/MS analysis (positive chemical ionisation mode) using a 1100 Series micro-LC chromatographic system (Agilent Technologies, Waldbronn, Germany) interfaced to a QTRAP® (Applied Biosystem/MSD Sciex, Foster City, CA, USA) equipped with a TurboIonSpray® interface. After derivatization with the labeling reagent (1-NC, 2-NC or PCC), a mixture containing 0.5 µg of each toxin, 20 µL of the reaction mixture were injected into the HPLC apparatus. Chromatographic conditions were as follows: analytical column, Phenyl-Hexyl Luna® (150 mm × 4.6 mm, 5 µm) (Phenomenex, Torrance, CA, USA); binary gradient, with the initial composition of the mobile phase, 70% acetonitrile:30% water containing 5 mM ammonium acetate, kept constant for 5 min, then the acetonitrile content was linearly increased to 85% in 10 min, and kept constant for 10 min; flow rate of the mobile phase, 1.0 mL min⁻¹ and splitting was performed to allow 100 µL min⁻¹ to enter the turbo-ion spray interface. Interface conditions were as follows: temperature, 300 °C; curtain gas (nitrogen), 20 psi; nebulizer gas (air), 10 psi; auxiliary gas (air), 30 psi; ionspray voltage, +4500 V.

Full scan experiments (Q1 scan mode, scan range 100–1000 amu) of the derivatization products were performed to analyze the presence of molecular ions or adducts with ammonium ion. HPLC–MS/MS analysis was carried out to find the characteristic fragment ions for each derivative.

2.6. Optimization of the excitation and emission wavelengths

Excitation and emission spectra of the new fluorescent derivatives were obtained with an Agilent 1100 Series HPLC system equipped with fluorescence detector (FD) (Agilent Technologies, Waldbronn, Germany). This programmable detector allowed the acquisition of fluorescence spectra during the chromatographic run and the determination of the optimum excitation and emission wavelengths of each chromatographic peak. After derivatization of a mixture containing 0.25 µg of each toxin with the labeling reagents (1-NC, 2-NC or PCC), 20 µL of the reaction mixture were injected twice into the HPLC/FD apparatus. The first injection was made to acquire excitation spectra (spectral ranges: $\lambda = 230\text{--}350\text{ nm}$ for 1-NC and 2-NC, $\lambda = 230\text{--}370\text{ nm}$ for PCC) in 2-nm steps by setting the emission wavelength at the maximum value of the labeling reagent (i.e. $\lambda_{\text{em}} = 380\text{ nm}$ for 1-NC, $\lambda_{\text{em}} = 370\text{ nm}$ for 2-NC and $\lambda_{\text{em}} = 405\text{ nm}$ for PCC). The second injection allowed the acquisition of emission spectra (spectral ranges: $\lambda = 280\text{--}650\text{ nm}$ for 1-NC and 2-NC, $\lambda = 380\text{--}650\text{ nm}$ for PCC) in 2-nm steps by using the optimum excitation wavelength previously determined for each fluorescent derivative.

2.7. Optimization of stoichiometric ratios

Different stoichiometric ratios of toxin:derivatizing reagent:catalyst were tested for 1-NC and 2-NC (1:10:20; 1:20:40; 1:40:80; 1:60:120; 1:80:250; 1:160:250), and for PCC (1:10:20; 1:20:40; 1:40:80; 1:60:120; 1:60:250; 1:120:500; 1:250:1000) in order to optimize the reaction yield. A mixture containing 0.7 µg of T-2 and 0.7 µg HT-2 toxins (corresponding to the immunoaffinity column capacity) was derivatized with each of the three labeling reagents in triplicate experiments.

2.8. Sensitivity, linear response and stability of T-2 and HT-2 derivatives

In order to test the fluorescence intensities of T-2 and HT-2 derivatives obtained with 1-NC, 2-NC and PCC and to compare them to the previously reported 1-AN derivatives [17], mixtures containing 0.25 µg of T-2 and 0.25 µg HT-2 toxin, were evaporated to dryness under a nitrogen stream and derivatized with: (a) 50 µL of DMAP solution (0.63 µg µL⁻¹) and 50 µL of 1-NC or 2-NC solution (0.49 µg µL⁻¹); (b) 50 µL of DMAP solution (2.00 µg µL⁻¹) and 50 µL of PCC solution (1.00 µg µL⁻¹); (c) 50 µL of DMAP solution (0.33 µg µL⁻¹) and 50 µL of 1-AN solution (0.30 µg µL⁻¹). Experiments were carried out in triplicate.

To evaluate the linearity of the response for T-2 and HT-2 derivative-peak areas, equimolar amounts of the two toxins (equivalent to 10–1000 ng of each toxin) were placed in screw-cap amber vials and the solvent evaporated to dryness at about 50 °C under a stream of nitrogen before derivatization.

In order to establish the stability of the fluorescent derivatives, different amounts of T-2 and HT-2 toxins (from 0.1 to 0.7 µg) were derivatized with 1-NC, 2-NC and PCC. The reaction mixtures were reconstituted with acetonitrile/water (70:30, v/v) and stored at –20, +4 and +25 °C in amber vials (‘dark’) and at +25 °C in clear vials (‘light’) for up to two weeks (triplicate experiments). At selected time intervals, 20 µL of the reaction mixture were injected into the HPLC apparatus as described above.

2.9. Analysis of cereal samples

Naturally contaminated samples of wheat, maize, and spiked oats were analyzed for T-2 and HT-2 toxins by HPLC/FD using either PCC or 2-NC as the pre-column derivatization reagent. Sample preparation was performed according to the method described by Visconti *et al.* [17], with minor modifications. Fifty grams of cereal sample, finely ground with a Model MLI-204 Bühler (Milan, Italy), were weighed into a blender jar, added with 1 g NaCl, and extracted with 100 mL methanol/water (90:10, v/v) by blending at high speed for 2 min with a Sorvall Omnimixer (Dupont Instruments, Newtown, CT, USA). The extract was filtered through filter paper. Ten millilitres of filtrate were collected and mixed with 40 mL of distilled water. For oat samples it was necessary to adjust the pH of the diluted extract at 7.4 value with NaOH. This additional step eliminated the presence of colloidal species in the purified extract that otherwise

caused interfering peaks in the chromatogram at the retention times of the T-2 and HT-2 derivatives. The diluted extract was then filtered through a glass microfibre filter. Ten millilitres of filtrate (equivalent to 1.0 g sample) were passed through the immunoaffinity column at a flow rate of about one drop per second, followed by 10 mL of distilled water at a flow rate of one to two drops per second. T-2 and HT-2 toxins were then eluted with 1.5 mL methanol and collected in a 4-mL screw-cap amber vial. The eluted extract was evaporated under a stream of air at ca. 50 °C, and the dried residue was derivatized with PCC or 2-NC as reported above.

3. Results and discussion

Three commercially available fluorescent reagents for modifying alcohols, 1-naphthoyl chloride (1-NC), 2-naphthoyl chloride (2-NC) and pyrene-1-carbonyl cyanide (PCC), were tested for labeling T-2 and HT-2 toxins (Fig. 1). All labeling reagents reacted with T-2 and HT-2 toxins under mild conditions to form the corresponding fluorescent esters. With respect to the derivatization procedure used for 1-anthroynitrile (1-AN) [17], the derivatization reaction was optimized. In particular, by performing the reaction while drying at 50 °C under a gentle stream of nitrogen in 10 min, instead of 15 min of reaction in solution at 50 °C followed by 10 min of cooling step in ice and solvent evaporation. Under these conditions the rate of reaction was increased by the effect of the increasing toxin concentration during the drying step and the cooling step, generally used to block similar esterification reaction, was avoided [13–15]. The dried mixture was directly reconstituted with acetonitrile/water (70:30, v/v) and injected in the HPLC/FD apparatus.

The identity of the T-2 and HT-2 esters [T-2(1-N); HT-2(1-N)₂; T-2(2-N); HT-2(2-N)₂; T-2(PC); HT-2(PC)₂] was confirmed by HPLC-MS and HPLC-MS/MS analysis (positive chemical ionization) of the reaction mixtures. In order to increase the sensitivity, 5 mM ammonium acetate was added to the mobile phase, allowing the detection of molecular ions as ammonium adducts [M+NH₄]⁺. Full scan chromatogram of the reaction mixture with 1-NC revealed the presence of a molecular ion with *m/z* 638.3 at the T-2 derivative retention time, corresponding to the adduct [T-2(1-N)+NH₄]⁺, and

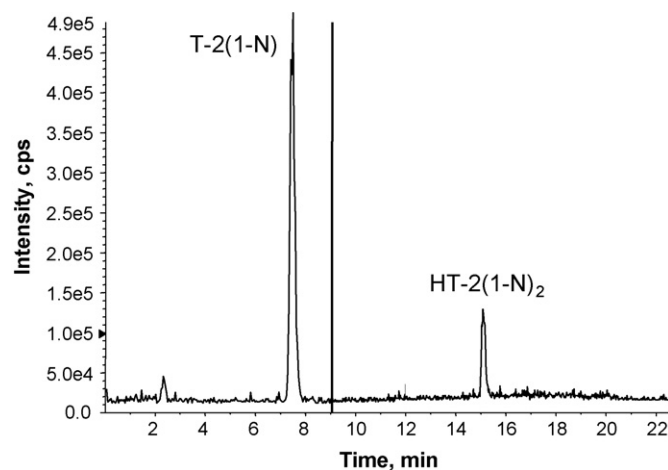


Fig. 2. HPLC-MS/MS chromatogram of T-2 and HT-2 toxins after reaction with 1-NC. Fragmented ions: *m/z* 638.3, [T-2(1-N)+NH₄]⁺ (0–9 min); *m/z* 750.3, [HT-2(1-N)₂+NH₄]⁺ (9–22 min).

a molecular ion with *m/z* 750.3 at the HT-2 derivative retention time, corresponding to the adduct [HT-2(1-N)₂+NH₄]⁺. No peak relevant to the mono-derivatized product for HT-2 toxin was observed. The HPLC-MS/MS chromatogram relevant to [T-2(1-N)+NH₄]⁺ and [HT-2(1-N)₂+NH₄]⁺ is shown in Fig. 2. In order to confirm the identity of the 1-NC derivatives, HPLC-MS/MS spectra were performed and compared with those of T-2 and HT-2 standards. Both MS/MS spectra of the ions [T-2(1-N)+NH₄]⁺ (*m/z* 638.3) and [HT-2(1-N)₂+NH₄]⁺ (*m/z* 750.3) showed a main fragment due to 1-naphthoyl group (*m/z* 155.2), and additional fragments at *m/z* 245.3, 227.3, 215.3, 197.3 and 185.0, respectively, originating from the trichothecene moiety. Similar results were obtained with the 2-NC derivatives. Full scan chromatogram of the PCC reaction mixture revealed the presence of a molecular ion with *m/z* 712.3 at the T-2 derivative retention time, corresponding to the adduct [T-2(PC)+NH₄]⁺, and a molecular ion with *m/z* 898.3 at the HT-2 derivative retention time, corresponding to the adduct [HT-2(PC)₂+NH₄]⁺. Also in this case no peak relevant to the mono-derivatized product for HT-2 toxin was observed. Both MS/MS spectra of the ions [T-2(PC)+NH₄]⁺ (*m/z* 712.3) and [HT-2(PC)₂+NH₄]⁺ (*m/z* 898.3) showed a main fragment due to pyrene-carbonyl group (*m/z* 229.2), and the characteristic fragments of the T-2 and HT-2 toxin moiety (i.e. *m/z* 227.3, 215.3, 197.3 and 185.0). Fig. 3 shows the scheme of the derivatization reaction of T-2 and HT-2 toxins with the labeling reagents 1-NC, 2-NC and PCC.

In order to establish the optimum excitation and emission wavelengths of each T-2 and HT-2 toxin derivatives for the HPLC-FD analysis, excitation and emission spectra were recorded during the chromatographic run of each fluorescent derivative. Table 1 shows the optimum wavelengths of excitation (λ_{ex}) and emission (λ_{em}) selected for each fluorescent derivative to improve sensitivity and selectivity of the HPLC analysis.

Different stoichiometric ratios of toxin:derivatizing reagent:catalyst were tested in order to improve the reaction

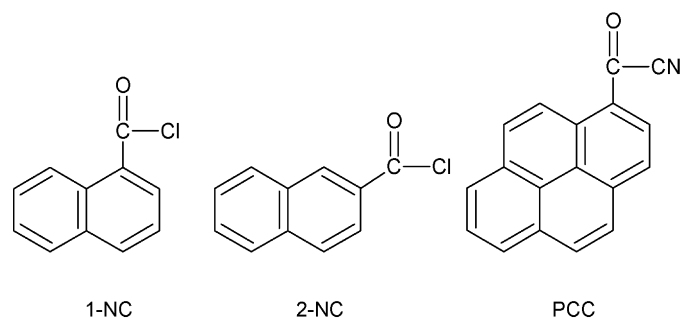


Fig. 1. Chemical structures of 1-naphthoyl chloride (1-NC), 2-naphthoyl chloride (2-NC) and pyrene 1-carbonyl cyanide (PCC).

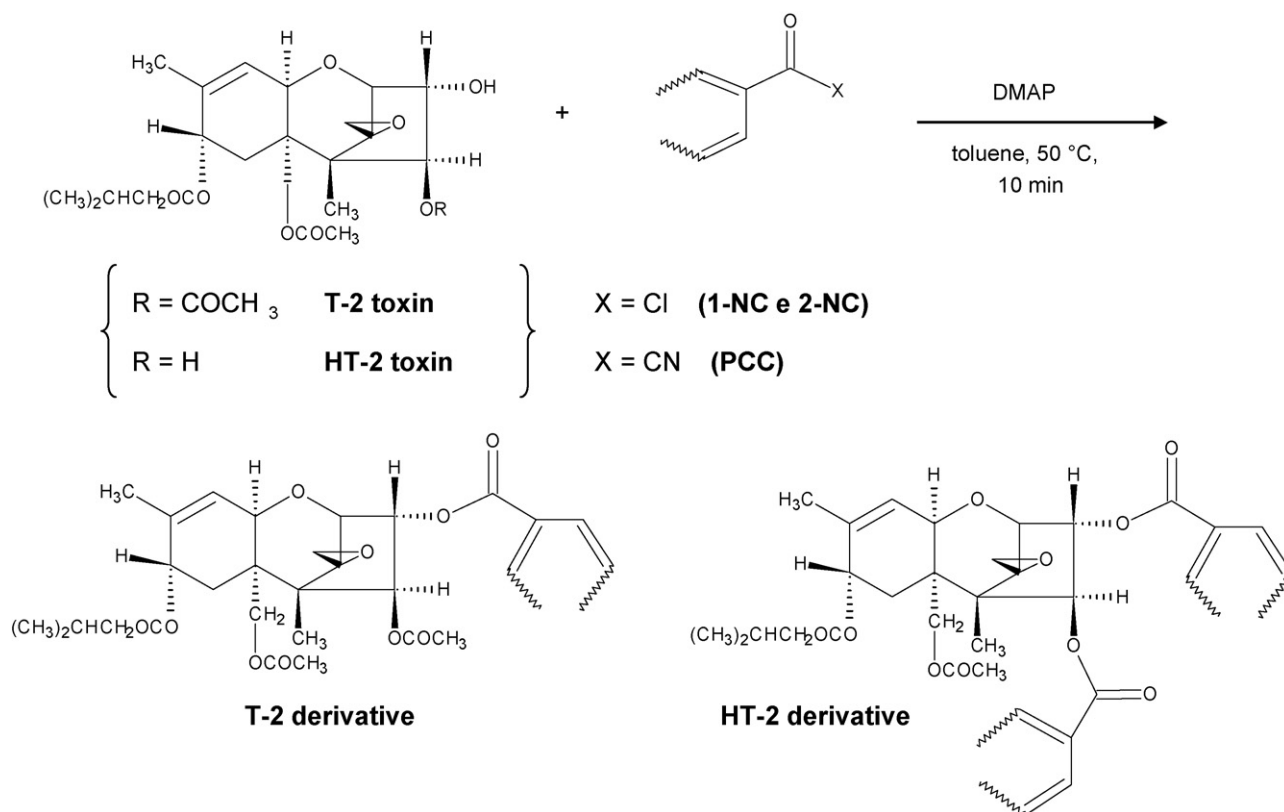


Fig. 3. Reaction of derivatization of T-2 and HT-2 toxins with 1-NC, 2-NC or PCC labeling reagents.

yields and to find the optimal conditions in terms of sensitivity and repeatability of the derivatization reaction for each labeling reagent. Fig. 4 shows fluorescence intensities of T-2 and HT-2 derivatives obtained at different stoichiometric ratios for 1-NC, 2-NC and PCC. No significant differences (ANOVA, test di Tukey, $p < 0.05$) were observed between peak areas obtained with ratios higher than 1:20:40 for HT-2(1-N)₂ and 1:40:80 for T-2(1-N), T-2(2-N), HT-2(2-N)₂ and T-2(PC). For HT-2(PC)₂ no significant difference ($p < 0.05$) was observed with ratios higher than 1:60:250. The PCC showed the highest reactivity towards both toxins, although the observed fluorescence intensity values of HT-2(PC)₂ derivative were unexpectedly lower than those of T-2(PC) at all tested stoichiometric ratios. Despite the apparent low reaction yield due to the low HT-2(PC)₂ signal, no peak corresponding to the unreacted HT-2 toxin was observed by HPLC-MS/MS

analysis under the optimized stoichiometric ratio conditions. The low fluorescence signal intensity can be explained by the intramolecular excimer formation involving the two pyrene rings of HT-2(PC)₂ producing change in fluorescence spectral profile and lifetime [18]. The best compromise taking into account noise, peak intensity of reagents and sensitivity for both derivatized toxins was obtained by using stoichiometric ratios of 1:40:80 for 1-NC and 2-NC reagents, and 1:60:250 for PCC reagent.

In order to evaluate the sensitivity of the new T-2 and HT-2 derivatives with respect to derivatives obtained by reaction with 1-AN [17], fluorescence intensities and signal-to-noise ratio values were determined by reacting the same amounts of toxins (250 ng of each toxin) under the optimized reaction conditions. Fig. 5 shows typical chromatographic profiles of derivatized T-2 and HT-2 toxins obtained by pre-column derivatization reaction with 1-NC, 2-NC and PCC. Detection limits (based on a signal-to-noise ratio of 3:1) were 10.0, 6.3 and 2.0 ng for derivatized T-2 toxin and 6.3, 2.3 and 2.8 ng for derivatized HT-2 toxin with 1-NC, 2-NC and PCC, respectively. As compared to detection limits of derivatized T-2 and HT-2 toxin with 1-AN (i.e. 5 and 3 ng, respectively), the sensitivity for both T-2 and HT-2 toxins was similar or better with 2-NC and PCC and worse with 1-NC.

A good linear response (peak area vs. injected amount) was observed, for all labeling reagents, in the range 10–1000 ng for either T-2 and HT-2 toxin, with coefficients of correlation (r) higher than 0.9995. Repeatability (relative standard devi-

Table 1
Optimum detection wavelengths of T-2 and HT-2 derivatives

Derivative	λ_{ex} (nm)	λ_{em} (nm)
T-2(1-N)	230	380
HT-2(1-N) ₂	230	376
T-2(2-N)	237	370
HT-2(2-N) ₂	237	365
T-2(PC)	355	403
HT-2(PC) ₂	355	401

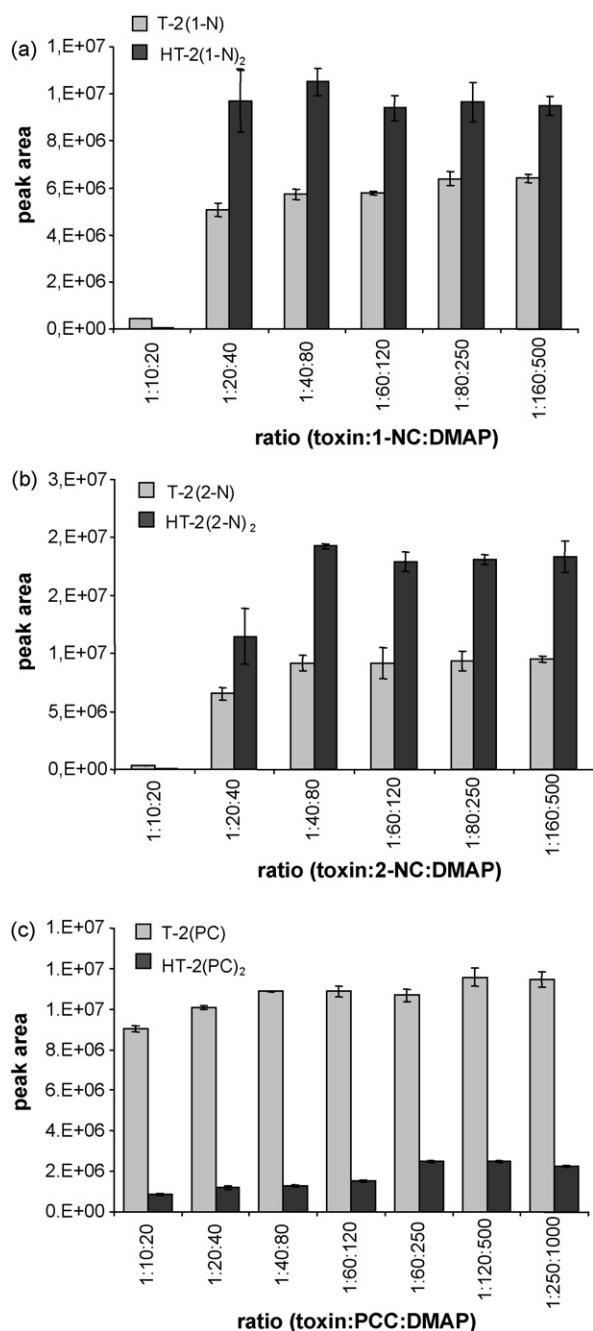


Fig. 4. Fluorescence intensities (peak areas) of T-2 and HT-2 derivatives obtained at different stoichiometric ratios of toxin:derivatizing reagent:catalyst for 1-NC (a), 2-NC (b) or PCC (c). Error bars represent relative standard deviations (triplicate experiments).

ations) of reactions was better than 8% for the derivatization of T-2 and HT-2 toxins over the whole concentration range tested.

Experiments to test the stability of the new T-2 and HT-2 fluorescent derivatives showed no significant difference of peak intensities up to 14 days after preparation of the solutions and storage at -20°C in amber vials ('dark') and up to 5 days at $+25^{\circ}\text{C}$ in clear vials ('light'), with the exception of T-2(PC) that showed highest stability under all the tested storage conditions.

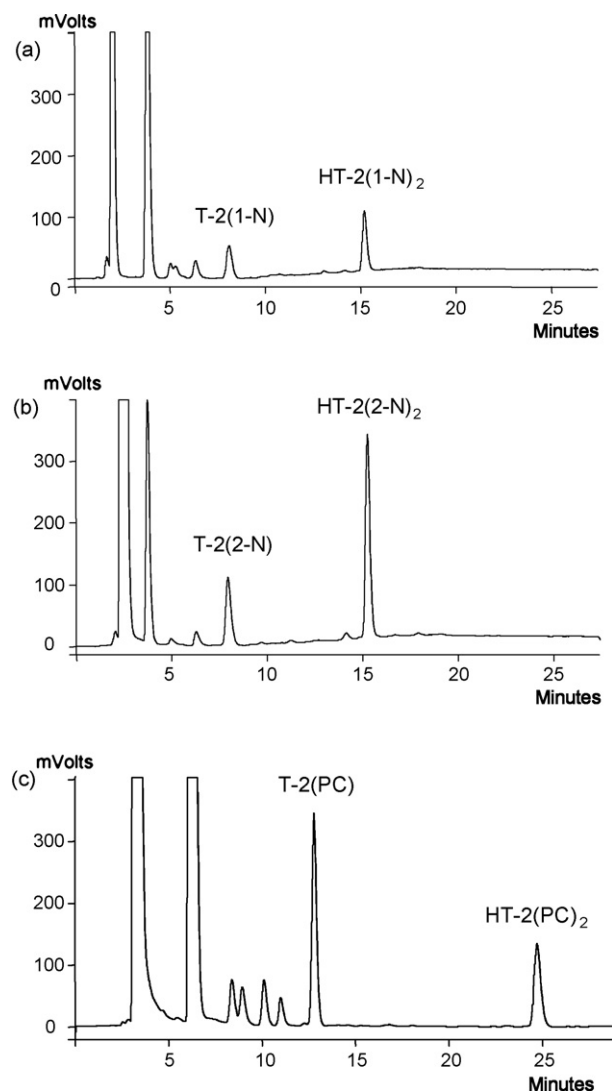


Fig. 5. Chromatograms of T-2 and HT-2 toxins (250 ng of each toxin,) derivatized with 1-NC (a), 2-NC (b) or PCC (c). Chromatographic conditions are reported in Section 2. All reaction mixtures were diluted (1:10, v/v) before HPLC injection to avoid off-scale peaks.

The high stability of the derivatives at room temperature would allow the use of an automatic sampler coupled with HPLC for the analysis of a large number of samples.

PCC and 2-NC reagents showed better performances in terms of the sensitivity of derivatives and the repeatability of reactions than 1-NC. In order to assess the applicability of the developed derivatization procedures with PCC and 2-NC for the simultaneous determination of T-2 and HT-2 toxins in cereals by HPLC/FD, preliminary experiments were carried out. Fig. 6 shows typical chromatograms of naturally contaminated wheat and maize extracts obtained by the procedure of Visconti *et al.* [17], and analyzed by HPLC/FD and pre-column derivatization with 2-NC and PCC. Fig. 7 shows chromatograms of a uncontaminated (blank) oat sample and the same oat sample spiked with T-2 and HT-2 toxin at levels of 30 and 15 ng g^{-1} , respectively, after immunoaffinity column clean-up of extracts and pre-column derivatization with 2-NC.

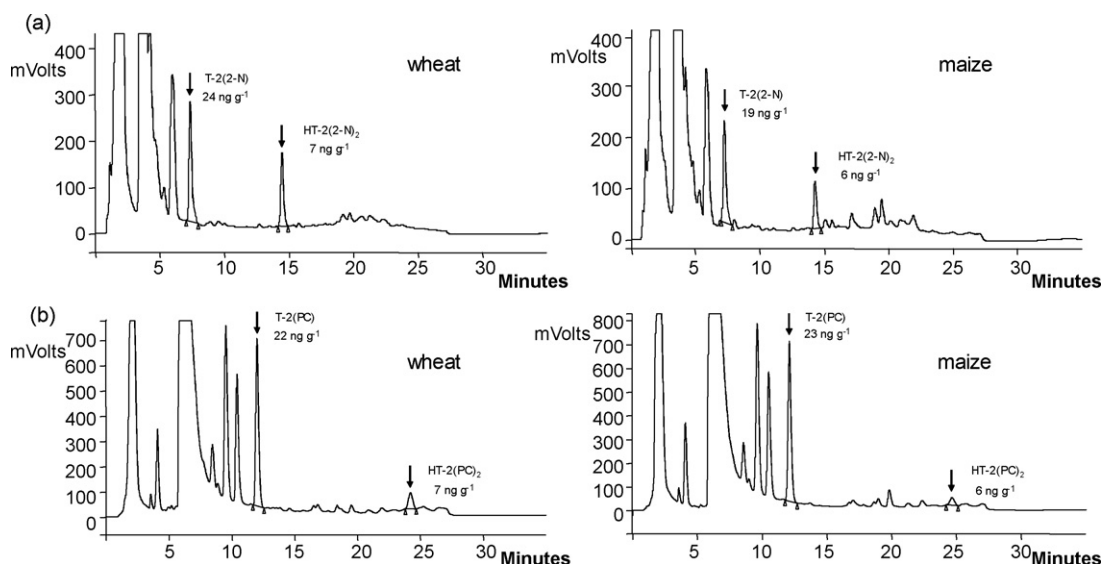


Fig. 6. Chromatograms of naturally contaminated wheat and maize samples after immunoaffinity column clean-up and pre-column derivatization with 2-NC (a) and PCC (b). Chromatographic conditions are reported in Section 2.

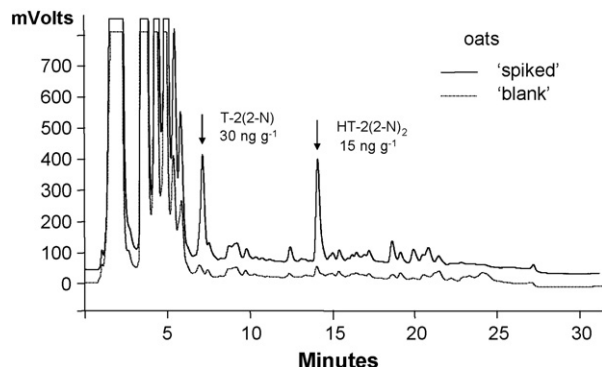


Fig. 7. Chromatograms of oat samples after immunoaffinity column clean-up and derivatization with 2-NC: (---) blank sample; (—) blank sample spiked with T-2 toxin (30 ng/g) and HT-2 toxin (15 ng/g). Chromatographic conditions are reported in Section 2.

4. Conclusions

1-NC, 2-NC and PCC, commercially available labeling reagents, reacted with T-2 and HT-2 toxins under mild conditions to give the corresponding fluorescent esters. The conditions of derivatization reactions were optimized in order to obtain good performance in term of rapidity, sensitivity, linearity, repeatability, and yield of reaction for each reagent. The use of 2-NC and PCC produced better performance, in term of sensitivity and selectivity, than 1-AN (a previously described labeling reagent for T-2 and HT-2 toxins) and 1-NC. Preliminary studies showed that derivatization reactions with 2-NC and PCC can be used for the simultaneous determination of T-2 and HT-2 toxins in raw cereals, including oats, by HPLC/FD after immunoaffinity column clean-up with good sensitivity. These novel fluorescent labeling reagents are useful tools to develop and validate sensitive and accurate analytical methods for determination of T-2 and HT-2 toxins in cereals and cereal-based products allowing a more correct evaluation of dietary exposure to these mycotoxins.

Acknowledgements

This work has been carried out within the project “Development of an improved method of analysis for the determination of T-2 and HT-2 toxins in foodstuffs” funded by the Food Standard Agency, UK. The authors would like to thank the National Research Council, Italy (Short-Term Mobility Program 2005) and V.M.T. Lattanzio (ISPA-CNR) for performing HPLC-MS analysis.

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